RESEARCH PLAN PROPOSAL

IN VITRO MORPHOGENETIC STUDIES AND PHYTOCHEMICAL ANALYSIS OF *SALVIA HISPANICA* (L.)

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INTRODUCTION

Chia (***Salvia hispanica* L.**) is a flowering plant of the mint family Lamiaceae, native to Central and Southern Mexico and Guatemala. The sixteenth century Codex Mendoza provides evidence that it was cultivated by the Aztec in pre-Columbian times; economic historians have suggested that it was as important as maize as a food crop (Cahill *et al*., 2003). It is still used in Mexico and Guatemala, in grounded form or whole seeds in nutritious drinks and food source (Kintzios *et al*., 2000).

Chia is an annual herb growing up to 1 m (3.3 ft) tall, with opposite leaves that are 4–8 cm (1.6–3.1 in) long and 3–5 cm (1.2–2.0 in) wide. Its flowers are purple or white and are produced in numerous clusters in a spike at the end of each stem. It is a short-day plant and commercially grown in Australia, Argentina, Colombia, Mexico and Peru.

Chia seed is a source of natural lipid antioxidants. Flavonol glycosides, cholorogenic acid and caffeic acid are found in chia extracts (Taga *et al*., 1984). The antioxidant activity of the fibre-rich fraction of chia flour was found to be higher than many cereals and similar to drinks such as wine, tea, coffee and orange juice (Vazquez *et al*., 2009). It is rich in fibre which becomes mucilagenous and expands considerably when soaked in water. The fibre consists of xylose, glucose and glucuronic acid (Lin and Daniel, 1994).

Chia seed contains about 20% proteins. The protein digestibility was evaluated by Torres *et al*. (2008). The digestibility was 79.8%, 34.2%, 29.1%, 24.3%, 10.9% in flour, toasted flour, raw seed, soaked seed, and toasted seed respectively. Chia flour was shown to have a low digestibility score which could be the influence of chia fibre.

Chia oil content ranges from 28.5 - 32.7% (Ayerza and Coates, 2004; Ayerza and Coates, 2007). Seeds of chia are rich source of omega-3 fatty acid and α-linolenic acid, (18:3). Chia diets dramatically decreased triacylglycerol levels and increased high density lipoprotein cholesterol and ω-3 fatty acid content in rat serum (Ayerza and Coates, 2005). Dietary chia seeds are also known to improve adiposity and insulin resistance in dyslipeamic rats (Chicco *et al*., 2009). Diets supplemented with chia have been found to decrease risks from some 8 types of cadiovascular diseases, cancers and diabetes. It has been reported that chia diet decreased the tumor weight and metastasis number and also inhibited growth and metastasis in a murine mammary gland adenocarcinoma (Espada *et al*., 2007). Long term supplementation with chia attenuated a major cardiovascular risk factor and emerging factors safely beyond conventional therapy, while maintaining good glycemic and lipid control in people with well-controlled type 2 diabetes (Kreiter, 2005; Vuksan *et al*., 2007 and Vuksan *et al*., 2009). Omega-3 fatty acids are reported to improve psychiatric disorders. There are reports where omega-3 fatty acids have been found effective in prevention and/or treatment of unipolar and bipolar depression (Freeman, 2006).

Cultivation of chia is confined to limited areas across the world. Rediscovery of this crop has increased its demand on a large scale. Tissue culture based strategies could be powerful tools for mass propagation of this wonder plant. Moreover, this technique could circumvent some of the constrains of conventional cultivation like season dependency, low germination rates of seeds etc. Thus, there is an urgent need to develop a rapid, reliable protocol for regeneration of this plant, since there is a single report on tissue culture of this plant.

In vitro technology has often used as a promising avenue to plant improvement for research and practical applications. It serves as a means of clonal propagation of economically important species and as an adjunct to traditional means of plant modification could reinforce the system thus providing a momentum to the process of wider implementation of the improved technologies

When plant tissue is passaged through *in vitro* cultures, many regenerated plants appear to be no longer clonal copies of their donor genotype. Among the factors that affect these tissue culture induced variations are explant genotype, explant tissue origin, medium composition and the length of time in culture duration. Variation is understood to be generated via a combination of genetic and/or epigenetic changes. A lack of any phenotypic variation between regenerants does not necessarily imply a lack of genetic (or epigenetic) change, and it is therefore of interest to assay the outcomes of tissue culture at the genotypic level. These *in vitro* raised culture variants can be characterized at molecular level. Changes in genome organization can be revealed by a rapid and unspecific DNA fingerprinting method like the Random Amplified Polymorphic DNA (RAPD) analysis which is a simple method to characterize genetic diversity and clonal fidelity without the need of detailed sequence information about the genomes of interest.

RAPD analysis is a quick and economic technique to identify genetic similarity among genotypes. The pattern of amplified bands obtained by RAPD could be used for clonal fidelity. RAPD based approach is capable of describing the qualitative and quantitative characteristics of the tissue culture induced variation.

The potential of higher plants as source for new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller. Thus, there is an urgent need to search for new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increase in the incidence of new infectious diseases as well as development of resistance to the antibiotics in current clinical use. The development and use of new phytochemicals has increased because of the appearance of resistant strains and attention has turned to natural antimicrobial agents in recent years (Daferea *et al*., 2003). Since chia plant is relatively unexplored with respect to its phytochemical and antimicrobial properties so there is immense scope of unravelling the therapeutic properties of this plant.

Fig: A twig of *Salvia hispanica*

Fig: Seeds of *Salvia hispanica*

Objectives

Keeping in view the above perspective following objectives have been proposed for the research work.

- Standardization of an efficient and reproducible micropropagation protocol for *Salvia hispanica* (L.)
- Acclimatization and Hardening of regenerated plantlets.
- Field transfer of regenerated plantlets.
- Evaluation of clonal fidelity of micropropagated plantlets using RAPD analysis.
- Preparation of plant extract of *Salvia hispanica* (L.) using various solvents.
- Evaluation of antimicrobial activity of plant extract.

Review of Literature

Chia being a very important plant in terms of nutritional value needs to be micropropagated through tissue culture. Establishment of an efficient plant tissue culture technique is a prerequisite for the production large number of plants in a limited period of time and can also circumvent the constraints of climatic conditions.

The present review briefly summarizes the micropropagation work carried out in some important plants of family Lamiaceae, molecular analysis using RAPD technique and antimicrobial activity.

In vitro behaviour of different *S. hispanica* explants was evaluated by using young leaves, cotyledons and uninodal segments with 2 axillary buds. The explants were inoculated on Murashige and Skoog (1962) (MS) culture medium, with 0, 1 μM of naphthalene acetic acid (NAA); 0, 1 μM of gibberellic acid (GA_3) and 0; 0.5; 0.75 or 1 μM of 6-bencyladenin (BA). The highest percentage of nodal segments with shoots was of 78%. A better survival of shoots and a higher proliferation of leaves/plantlet were observed with 0.75 and 1 μM of BA. (Bueno *et al*.2010)

Nodal explants of *Salvia officinalis L.* grown on MS media induced adventitious shoots regeneration. The highest rate of growth was achieved on MS medium supplemented with BAP (2.22 μM) and with Kn (4.65 μM) and NAA (2.68 μM). The rooting process could be initiated only on Kn medium (Irina, 2008). . Low light intensities (50 mmol m^{-2} s^{-1}) favoured callus formation and induction of somatic embryos of *Salvia officinalis* on a MS medium supplemented with 1.8–1.8 mM 2,4-D and Kn, 10.5–21 mM NAA and BA or 1mM TDZ. (Boldura *et al*. 2010)

Makunga and Staden (2008) developed an *in vitro* cultivation protocol for *S. africana-lutea*. Adventitious shoot induction was most successful using hypocotyls as explants for propagation on MS medium supplemented with 4.4 μM BA only; 2.7 μM NAA and 4.4 μM BA; or 2.9 μM IAA and 9.3 μM kinetin respectively. For continuous subculture, IAA and BA (μM) at a ratio of 2.9:4.4 or 2.9:8.9 had the best regeneration potential producing approximately three plantlets per nodal explant.

Rapid multiplication of *Salvia canariensis* L. from axillary node on MS + 825 mg/l NH_4NO_3 - supplemented with 10^{-7} M BA and 10^{-7} M NAA. Well developed root systems was produced within three weeks after transfer to the

same culture medium supplemented with 5x 10^{-7} M NAA (Molina *et al*. 1997). Stem and petiole explants were cultured on MS and B5 culture media supplemented with BAP, NAA and IBA for induction of callus and shoot buds. However, IBA fortied medium inhibited shoot induction. The shoots developed from both the explants formed well developed root system when transferred to half strength MS medium supplemented with IAA, NAA or IBA. (Molina 2004)

Fadel *et al*. (2010) investigated the effect of inorganic salt concentration on the *in vitro* organogenesis of spearmint (*Mentha spicata* L.) by using MS medium of different strength (full, half and quarter strength). The highest number of shoots and roots induced per explant (3.5 and 10, respectively), as well as the maximum average shoot length (16 cm) was observed on half strength medium. The maximum leaf number (35 per explant) and average root length (7 cm) were associated with regeneration on full medium. Highest frequency of meristemoids and morphogenetic callus were initiated from explants of *Mentha spicata* (L.) cultured onto a MS medium salts, supplemented with 4 mg/l TDZ and 25% (vol/vol) CW for 10 to 14 d in darkness. Bud and shoot development required removal of both TDZ and CW from the medium. (Li *et al*. 1999).

Rapid multiplication of *Mentha piperita* L. through shoot tip and nodal explants on MS medium supplemented with either BAP; 1 mg/l or zeatin (0.25 mg/l) show highest number of shoots (49.8) obtained on medium containing BAP. The regenerated dwarf shoots were further elongated on MS medium supplemented with GA_3 (1 mg/l). *In vitro* shoots were then excised from shoot clumps and transferred to rooting medium containing NAA (1 mg/l). (Ghanti *et al*. 2004) . According to Manik *et al*. (2012) shoot tip, nodal and sucker explants exhibited highest number of shoots i.e. 4.25±0.85 at Kn (1.5 mg/l) and highest shoot length i.e. 4.72±0.87 cm. Highest number of roots (6.5±2.67) and highest root length (5.07±1.22 cm) was observed at IAA 2 mg/l and IBA 2 mg/l. Shoot

regeneration on 1/2 strength MS salts and vitamins supplemented with various concentrations of BAP, Kn alone or with NAA was also achieved. However, internode and petiole explants were not potent and failed to regenerate shoots at some of the tested plant growth regulators concentrations. Variable frequency of rooting was recorded at four different concentrations of auxins. (Sarwar *et al*. 2009)

Amutha *et al*. (2008) obtained a direct organogenesis and *in vitro* flowering in *Basilicum polystachyon* (L.) Moench. High frequency and maximum number of multiple shoots were obtained from shoot tip explants on MS medium supplemented with BAP (2.22-13.32µM) and Kn (2.32-13.92µM).Regenerants, when transferred to rooting medium IBA (2.46-14.76µM) and IAA (2.85-17.13 µM), initiated flowering along with rooting.

The effects of explants source of O*cimum basilicum* L. (sweet basil) on MS medium supplemented with four different concentrations of BAP and second, the effects of different levels of two growth regulators (BAP and IAA) either individually or in combination on multiple shoot induction from nodal segments was evaluated by Farhad *et al*., 2012. Maximum percent of regeneration (96.67±0.33) and average number of shoot (5.6±1.15) were observed on the medium containing 11 µM BAP + 0 µM IAA. Similarly, increasing BAP concentration led to decreased rooting. The lowest and the highest vitrification values were achieved on the media containing 0 and 33 µM BAP.

Axillary shoot bud proliferation was initiated from nodal explants of *Ocimum gratissimum* cultured on MS medium supplemented with various concentrations of BA (0.5 - 3.0 mg/l), Kn (0.5 - 3.0 mg/l) and 2-iP (0.5 - 3.0 mg/l). Maximum numbers of shoots (5.17 ± 0.04) with average length (2.50 ± 0.07) were induced on medium containing 1.0 mg/l BA. Rooting of shoots was achieved on half

strength MS medium supplemented with 1.5 mg/1 IBA and 2% sucrose. Genetic fidelity was assessed by chromosome analysis and DNA fingerprinting using random amplified polymorphic DNA (RAPD) of *in vitro* and *in vivo* plants. Nine arbitrary decamers displayed same banding profile showed no genomic alterations, indicating homogeneity among the tissue culture regenerates and genetic uniformity with that of donor plants. (Saha *et al*. 2012).

RAPD analysis is a quick and economic technique to identify genetic similarity among genotypes. The pattern of amplified bands obtained by RAPD could be used for genomic fidelity (Welsh and McClelland, 1990). RAPD based approach is capable of describing the qualitative and quantitative characteristics of the tissue culture-induced variation.

Cahill, (2004) investigated genetic diversity among 38 wild and domesticated accessions of *S. hispanica* collected by using RAPD markers. Genetic diversity was higher among wild varieties (H(G) = 0.15) than all domesticated varieties (H(G) = 0.10) and modern commercial domesticated varieties (H(G) = 0.02), suggesting a slight loss of diversity accompanying domestication and a near lack of diversity in modern commercial varieties. The centre of genetic diversity was found in the highlands of western Mexico.

The phylogenic relationship among four species of *Salvia* (*S. macrosiphon* Boiss*., S. Aethiopis* L*., S. brachyantha* (Bordz.) Pobed. and *S. sclarea* L.) and one species of *Nepeta* (*N. saccharata* Bunge) was investigated by RAPD analysis. All the species were clearly identified using 20 primers tested (Z1-Z20). It indicated that *N. saccharata* Bunge had the closest relationship with two samples of *S. macrosiphon* and *S. Aethiopis*. The species *S. brachyantha* have not only showed farther genetic distance from *S. macrosiphon* and *S. Aethiopis* but also different patterns of RAPD-PCR compare to others.

Dendrogram showed one main cluster included five tested species of *Salvia* and *Nepeta* (Saeidnia *et. al.*2009).

The essential oil composition and genetic variability of common sage (*Salvia officinalis* L.) and its three ornamental cultivars ('Purpurascens', 'Tricolor', and 'Kew Gold') as well as Judean sage (*Salvia judaica Boiss*.) were analyzed by GC-FID, GC-MS, and RAPD. Common sage and its cultivars contained the same volatile compounds; only the ratio of compounds differed. The main compounds were the sesquiterpene R-humulene and the monoterpenes β-pinene, eucalyptol, and camphor. Judean sage contained mainly the sesquiterpenes β-cubebene and ledol. All of the samples exhibited characteristic RAPD patterns that allowed their identification. (Boszormenyi *et.al.*2009)

Some species have been studied for their antimicrobial properties attributed to its essential oils such as *Salvia sclarea*, which have isolated four diterpenes abiatanos: salvipisona, aetiopinona, 1-ferru-ginol oxoaetiopinona and its roots, this diterpene last abiatano also was obtained from *S. lavandulifolia* and *S. officinalis* pinene, cienolo and camphor. The clinical efficiency of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens (Bandow *et al.*, 2003).The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity.

The water-distilled essential oil of sage (*Salvia officinalis L.*) grown in Serbia was analyzed by GC/MS. The essential oil (2% and 1% in ethylene glycol) from the sage leaves was found to be active against *Bacillus subtilis S, Staphylococcus aureus* 6538, *Escerichia coli* 95, *Salmonella enteritidis and Aspergillus niger* (D. Miladinović, Lj. Miladinović, 2000).

Magda coisin *et al*., (2012) investigated essential oils of *Salvia aethiopis* L., *S. nemorosa* L. and *S. verticillata* L. in terms of chemical composition and antibacterial activity and tested on two pathogen bacterial strains: *Staphylococcus aureus* and *Escherichia coli.* Essential oils obtained by hydro-distillation were analysed using GC-MS method. The minimal inhibitory concentration (MIC) was determined by diffusion method and successive micro-dilutions. Test results have shown a greater antibacterial effect of *Salvia* oil samples against Gram positive, than the Gram negative bacteria

The essential oils of *Salvia officinalis* and *Salvia triloba* cultivated in South Brazil were analyzed by GC–MS. The essential oils of both species exhibited remarkable bacteriostatic and bactericidal activities against *Bacillus cereus*, *Bacillus megatherium, Bacillus subtilis, Aeromonas hydrophila, Aeromonas sobria*, and *Klebsiella oxytoca*. Moreover, the essential oil of *S. triloba* efficiently inhibited the growth of *Staphylococcus aureus. S. aureus*and *A. hydrophila* growth were drastically reduced even in the presence of 0.05 mg/ml of the essential oil of *S. triloba* (Ana Paula Longaray Delamare *et al*.,2005).

Methodology

- **Collection and authentication of plant materials**

  Salvia hispanica (L.), an important flowering plant has been taken up for the present study. *Salvia hispanica* (L.) is a native to Central and Southern Mexico and Guatemala. The seeds would be procured from Namdhari Seed Agency, Bangalore.

- **Sterilization and Preparation of explants**

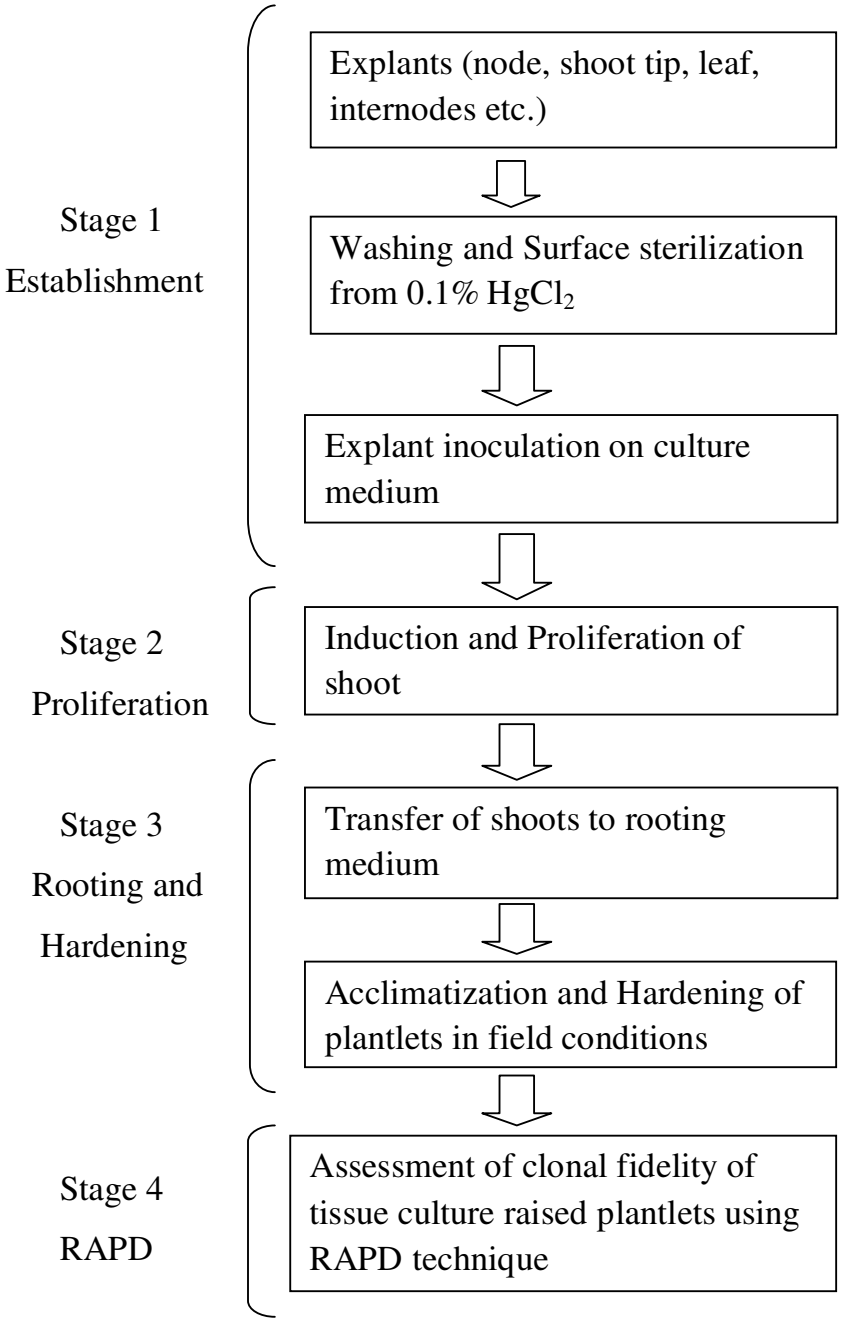
  Shoot tips and nodal segments will be used for induction of multiple shoots. Explants will be washed thoroughly under running tap water for 20 min. Later these explants will be sterilized with 0.1% $HgCl_2$ (w/v) for 5 min and washed thrice using sterilized distilled water. Under aseptic conditions, explants will be inoculated on MS medium.

- **Tissue culture of *Salvia hispanica* (L.) explants**

  Nodal explants and shoot tips would be inoculated on MS medium supplemented with various concentrations of cytokinins added singly or in combination with different auxins for regeneration of shoot buds from *Salvia hispanica* (L.). Plantlets with well developed root and shoot system would be acclimatized and hardened.

- **Clonal fidelity assessment using RAPD technique**

  DNA would be extracted from the tissue culture-derived plantlets. The extracted DNA would be subjected to amplification using PCR technique. RAPD analysis using arbitrary oligonucleotide primers would be employed in order to investigate the genetic fidelity of plant tissue culture-derived regenerants of *Salvia hispanica* (L.).

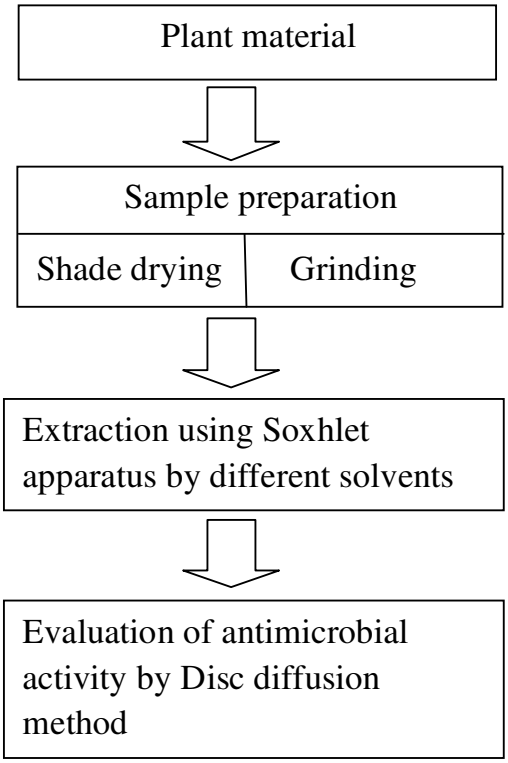

Stage 1
Establishment
Explants (node, shoot tip, leaf, internodes etc.)
Washing and Surface sterilization from 0.1% $HgCl_2$
Explant inoculation on culture medium
Stage 2
Proliferation
Induction and Proliferation of shoot
Stage 3
Rooting and
Hardening
Transfer of shoots to rooting medium
Acclimatization and Hardening of plantlets in field conditions
Stage 4
RAPD
Assessment of clonal fidelity of tissue culture raised plantlets using RAPD technique

- **Preparation of plant extract**

  The seeds of *Salvia hispanica* (L.) will be air-dried and pulverized in to course powder by using a dry grinder and passed through the sieve before being stored in closed vessel for further use. Dried seeds of *Salvia hispanica* (L.) will be powdered and weighed accurately and subjected to extraction in a soxhlet apparatus at room temperature for 48 hours using different solvents. The extract will be filtered and stored at 4 ° C for further analysis.

- **Evaluation of Antimicrobial activity**

  The antimicrobial activity of seeds of *Salvia hispanica* (L.) plant will be evaluated by Disc Diffusion method / MIC / Agar well Diffusion method on selected bacterial strains (Bauer and Kirby, 1961).



Significance of the work

Salvia hispanica (L.) is a marvellous plant in present times because it has numerous medicinal properties such as antiplatelet, anti-inflammatory, antidiabetic, antitumor and anticarcinogenic, antimutagenic, hypotensive, antimutagenic, antioxidant, or biomedical applications. Cultivation of chia is confined to limited area across the world thus there is an urgent need to develop a rapid, reliable protocol for regeneration of this plant which is a prerequisite for the production large number of *Salvia hispanica* (L.) plants in a limited period of time and can also circumvent the constraints of climatic conditions.

Plant tissue culture seems to be especially prone to genome variation. Changes in genome organization can be revealed by a rapid and unspecific DNA fingerprinting method like the RAPD analysis and it is a simple method to characterize genetic diversity and clonal fidelity without the need of detailed sequence information about the genomes of interest.

The clinical efficiency of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity. Thus, there is an urgent need to search for new antimicrobial compounds with diverse chemical structures and novel mechanisms of action.

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